POMC in $Vgf^{-/-}$ mice was downregulated, while that of NPY and AgRP was upregulated [39]. Additional phenotypes of $Vgf^{-/-}$ mice include delay of puberty, abnormal sexual behavior, small reproductive end organs, and shortening of the circadian rhythm. These reproductive abnormalities are thought to be associated with the suppression of gonadotropin-releasing hormone release from the hypothalamus and of sex hormones, follicle stimulating hormone, and lutenizing hormone from the pituitary [16]. The phenotypes of $Vgf^{-/-}$ mice suggest that VGF is an endogenous modulator of neuropeptide release in the hypothalamus.

Several types of VGF-derived peptides have been detected in the rat brain, bovine pituitary, and human cerebrospinal fluid [40–48]. Immunoblotting of PC12 cells or rat brain extracts using an antibody raised against the C-terminal region of VGF showed the presence of peptides with molecular weights of approximately 20 kDa (VGF20/NAPP-129), 10 kDa (VGF10/TLQP-62), and 2 kDa (VGF2/LQEQ-19 and AQEE-30) (Fig. 1B) [42, 44]. These VGF-derived peptides are detected in dense core secretory granules of neuronal and neuroendocrine cells [42, 44].

We demonstrated that NERP-2 colocalized with orexins, but not the melanin-concentrating hormone, in the lateral hypothalamus of rats. Icv administration of NERP-2 induced Fos, a marker of neuronal activation, in the orexin neurons. Icv administration to rats of NERP-2, but not NERP-1, enhanced food intake in an orexin-dependent manner (paper in submission).

These peptides possess multiple biological activities, functioning in synaptic plasticity, antidepression, penile erection, autonomic activation, and increases in energy expenditure [43, 44, 47, 48]. Icv administration of TLQP-21 increased energy expenditure and prevented diet-induced obesity in mice on a high fat diet [46] and decreased food intake in Siberian hamsters [49]. VGF-derived peptides are enriched in the secretory granules which are preferentially secreted upon cell membrane depolarization [43]. The storage of multiple species of VGF-derived peptides in secretory granules and widespread expression of VGF throughout the central and peripheral nervous systems and endocrine tissues are similar to the patterns seen for the chromogranin-secretogranin family [50]. Chromogranins and secretogranins, collectively known as "granins", are a unique group of acidic, soluble secretory proteins with molecular weights ranging from 21 to 67.5 kDa [50]. Granin-derived polypeptides regulate the secretion of other peptides in autocrine, paracrine, and endocrine fashions. The degree of similarity in the expression pattern and characteristic protein sequences of VGF with proteins from the chromogranin-secretogranin family suggests the potential involvement of NERPs in the regulation of peptide release.

Concluding remarks and future perspectives

In conclusion, VGF is synthesized exclusively in neuronal and neuroendocrine cells, and VGF-derived peptides appear to regulate the release of peptides or hormones via the regulated secretory pathway. NERPs are novel bioactive peptides involved in body fluid homeostasis that appear to modulate the actions and secretion of other neuropeptides in an autocrine, paracrine, or endocrine fashion. We demonstrated that NERP-2, but not NERP-1, stimulated feeding behavior in rats and mice. We also found that NERP-producing cells are widely distributed in the endocrine and neuroendocrine cells in systemic organs. Further studies of NERPs and their receptors will pave the way for elucidating unknown extracellular signaling mechanisms as well as understanding the roles of NERPs not only in body fluid homeostasis, but also in the regulation of various physiological phenomena.

Acknowledgements. We thank Drs. Hideki Yamaguchi, (Department of Internal Medicine, University of Miyazaki), Naoto Minamino and Kazuki Sasaki (Department of Pharmacology, National Cardiovascular Center Research Institute), Toshifumi Takao and Yoshinori Satomi (Laboratory of Protein Profiling and Functional Proteomics, Institute for Protein Research), Seiji Shioda and Haruaki Kageyama (Department of Anatomy, Showa University School of Medicine), and Luis J. Gonzàlez (Physical-Chemistry Division, Center for Genetic Engineering and Biotechnology, Habana, Cuba).

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ANTICANCER RESEARCH 29: 4211-4216 (2009)

Induction Chemotherapy with Docetaxel, 5-FU and CDDP (DFP) for Advanced Gastric Cancer

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Abstract. *Background: The aim of this study was to evaluate the feasibility and efficacy of modified triplet chemotherapy with docetaxel, 5-fluorouracil and cisplatin as induction chemotherapy for advanced gastric cancer (AGC). Patients and Methods: Treatment-naïve patients with AGC were eligible. The regimen consisted of 350 mg/m^2/day 5-FU by continuous infusion on days 1 to 5, 10 mg/m^2/day CDDP intravenously on days 1 to 5, and docetaxel at 60 mg/m^2/day intravenously on day 1. After 2 cycles (each cycle consisted of 4 weeks), surgical resection was attempted, 2-4 weeks after the completion of the regimen. Results: Eighteen patients were enrolled. Adverse events included grade 3 anorexia and nausea in 16.7% and 11.1% and grade 4 leukocytopenia and neutropenia in 5.6% and 27.8%, respectively. The overall response rate was 44.4%. Surgery was conducted in 15 patients. The 1- and 3-year survival rates were 75.6% and 51.1%, respectively. Conclusion: The modified triplet combination therapy is effective and well tolerated by patients with AGC.*

Although the incidence of gastric cancer is declining in Western countries, it is still the second most frequent cause of cancer-related death worldwide (1). Similar to other malignancies, the survival of patients with gastric cancer depends on the clinical stage of the disease. Surgery remains the treatment of choice for curing early-stage disease. On the other hand, the prognosis of patients with locally advanced or distant metastatic gastric cancer is still very poor even after surgery. Recent results of a randomized control trial showed that D2 lymphadenectomy plus extended para-aortic lymph node dissection provide no survival benefits compared to D2 alone (2), emphasizing the limited benefits of surgery for advanced gastric cancer (AGC). MacDonald *et al.* (3) have reported that postoperative adjuvant chemoradiotherapy significantly improved the relapse-free survival (RFS) and overall survival (OS) of patients with AGC compared with surgery alone. Furthermore, Cunningham *et al.* (4) reported in the results of the MAGIC trial that perioperative chemotherapy significantly improved both the RFS and OS of patients compared to surgery alone. These studies suggested that the selection of efficient perioperative chemotherapy for gastric cancer is important for the improvement of outcome of AGC.

Over the last decade, new active agents, including taxanes (paclitaxel (5, 6) and docetaxel (7, 8)), irinotecan (9), oxaliplatin (10), and S-1 (11, 12) have been developed and several randomized phase II/III studies have identified promising combination regimens for non-resectable cases of gastric cancer (13-18). Thus, in order to improve the rate of curative resection and to prolong the survival of patients after surgery, neoadjuvant chemotherapy (NAC) or induction chemotherapy should be investigated with chemotherapeutic regimens including novel active agents (19-22).

Docetaxel has shown promising activity when administered alone (response rate: 17-24%) (7, 8, 23, 24) or in combination with other agents (16, 25, 26). The phase III V325 study indicated that DCF (docetaxel, cisplatin and fluorouracil) was superior to CF (cisplatin and fluorouracil) in terms of response rate, time to progression, and OS (27). However, grades 3 to 4 treatment related adverse effects occurred in 82% and 57% of patients treated with DCF and CF, respectively (27). The original regimen of DCF in the V325 trial, was docetaxel at 75 mg/m^2 (1-hour intravenous infusion) plus CDDP at 75 mg/m^2 (1- to 3-hour intravenous infusion) on day 1, followed by 5-FU at 750 mg/m^2/day (continuous intravenous infusion) for 5 days every 3 weeks. To improve the feasibility of the triple-agent therapy, the dosage of docetaxel was reduced to 60 mg/m^2, which is the

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Key Words: Gastric cancer, neoadjuvant chemotherapy, docetaxel, 5-fluorouracil, cisplatin, DFP.

recommended dose in Japan (8, 23, 24). Furthermore, reduced dose 5-FU and split low-dose CDDP have been introduced to reduce the adverse events (28, 29). The aim of this study was to evaluate the toxicity and efficacy of a docetaxel, low-dose 5FU and split low-dose CDDP combination for AGC as an induction chemotherapy.

Patients and Methods

Patients. Patients with locally advanced and/or distant metastatic gastric cancer who were treated at Osaka University Hospital (Osaka, Japan) between October 2001 and January 2008 were enrolled in this study. Staging laparoscopy was performed for patients with serosa-invading gastric cancer to detect peritoneal dissemination. The inclusion criteria were as follow: age, 20-75 years; no prior chemotherapy; ECOG performance status, 1 -2 (30); existence of measurable target lesions by RECIST criteria (31); adequate function of major organs; no other active malignancy; estimated life expectancy of more than 3 months and provision of written informed consent. Patients were excluded if they were found to have severe co-morbid conditions, infectious diseases, brain metastasis, massive pleural effusion, massive pericardial effusion, peripheral neuropathy or a past history of drug allergy. Furthermore, pregnant and breast-feeding women were also excluded. The patients were classified according to the Japanese Classification of Gastric Cancer (32). The study protocol was approved by the Human Ethics Review Committee of Osaka University School of Medicine.

Treatment regimen. The regimen used for the treatment of the enrolled patients is illustrated in Figure 1. This regimen was repeated every 4 weeks for a total of 2 cycles. All the patients underwent hematological tests and physical examination before the start of each course. If the following toxicities occurred, the next administration was delayed until full recovery from the toxicity and the doses of all the drugs (docetaxel, 5-FU, and CDDP) were reduced by 25% in the following course: leukocyte count <3000/μl; platelet count <10.0×10⁴/μl or non-hematological toxicity of ≥grade 3. If complete resection was expected or the non-curative resection factor was liver metastasis only, surgery was attempted 2-4 weeks after the chemotherapeutic regimen. The primary end point was the overall response rate for chemotherapy, while the secondary end points were OS, the toxicity profile and the rate of complete resection.

Evaluation of toxicity, response and survival. Blood cell counts and blood chemistry (including liver and renal function tests) were performed at least once a week. The toxicity of the chemotherapy was monitored and graded according to the Common Toxicity Criteria of the National Cancer Institute version 2.0 (http://www.cancer.gov). The tumor response was assessed by computed tomography at every cycle of treatment and evaluated by the Response Evaluation Criteria in Solid Tumor (RECIST) (31). The RECIST criteria are defined as follows: complete response (CR), the disappearance of all target lesions; partial response (PR), at least a 30% decrease in the sum of the longest diameters of the target lesions, taking as reference the baseline sum of the longest diameters; progressive disease (PD), at least a 20% increase in the sum of the longest diameters of the target lesions, taking as reference the smallest sum of the longest diameter recorded since the treatment started or the appearance of one or more new lesions; stable

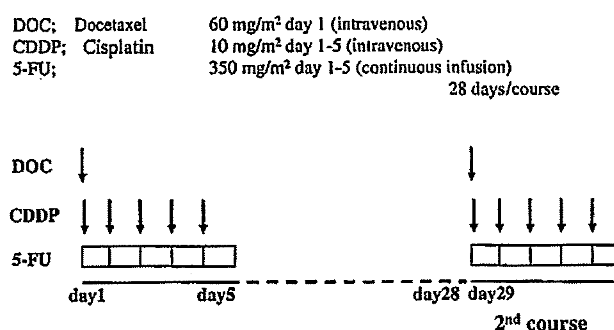


Figure 1. *Treatment regimen. The regimen was repeated every 4 weeks for a total of 2 cycles.*

disease (SD), neither sufficient shrinkage to quality for PR nor sufficient increase to quality for PD, taking as reference the smallest sum of the longest diameter since the treatment started.

Statistical analysis. Numerical values are expressed as the median (range). Survival was defined from the first day of chemotherapy to death from any cause and calculated by the Kaplan-Meier method. All the calculations were performed with the software package Statview Version 5.0 (SAS Institute, Inc, Cary, NC, USA).

Results

Patient characteristics. A total of 18 patients with AGC (adenocarcinoma) were enrolled in this trial. The Eastern Cooperative Oncology Group performance status was 0 or 1 in 16 (89%) patients. The reasons for induction chemotherapy were bulky N2 lymph node (LN) metastasis in 3 patients, N3 metastasis in 5 patients, tumor invasion of adjacent organs (T4) in 3 patients, liver metastasis in 3 patients, lung metastasis in 2 patients, distant LN metastasis in 1 patient and peritoneal dissemination in 1 patient. The patient characteristics are listed in Table I.

Adverse events. Eighteen patients received a total of 32 treatment cycles. The average number of cycles administered per patient was 1.8. Four patients received only one cycle of chemotherapy, two were due to tumor progression and two due to toxicity and deterioration of performance status. The most common adverse events were gastrointestinal toxicity, leukocytopenia and neutropenia. Grade 3 anorexia and nausea occurred in 16.7% and 11.1% of the patients, respectively. Grade 4 leukocytopenia and neutropenia occurred in 5.6% and 27.8%, respectively. The adverse events are summarized in Table II.

Response to induction chemotherapy. None of the 18 enrolled patients showed a CR, while 8 showed PR, 8 showed SD, and 2 showed PD by the RECIST criteria. The overall response rate was 44.4%. The response rate in the intestinal type primary tumors was 33.3% and in the diffuse type was

Table I. *Patient characteristics.*

n		18	
Median age (range)		57 (35-75)	
Male/Female		15/3	
ECOG-PS 0/1/2		4/12/2	
Borrman type 1/2/3/4		1/6/8/3	
Histopathological type Intestinal/diffuse6/12			
Localization U/M/L		6/5/7	
cStage IIIB/IV		3/15	
Non-curative resectable factor			
Bulky N2	3	N3	5
T4	3	H1	3
Lung	2	P1	1
Distant LN	1		
Mean no. of treatments (range)		1.8 (1-2)	

ECOG: Eastern Cooperative Oncology Group, PS: performance status, LN/N: lymph node, U: upper third portion of the stomach, M: middle third portion of the stomach, L: lower third portion of the stomach, T4: tumor invasion of adjacent structures, H1: liver metastasis, P1: peritoneal dissemination.

50.0%. The response rate for each target organ is listed in Table III. Histopathological examination showed no residual tumors (grade 3) in resected specimens of one patient.

Surgery. Gastrectomy was conducted in 15 out of the 18 patients. Surgery was considered curative in 11 patients and non-curative in 4 patients. The two patients with lung metastasis and one patient with distant lymph node metastasis were excluded. Total gastrectomy was performed in 8 patients, distal gastrectomy in 6 patients, and pancreato-duodenectomy in one patient due to tumor spread to the pancreatic head. Extended surgery was conducted in 10 patients: para-aortic lymphadenectomy in 6 patients, partial hepatectomy in 2 patients, left pancreatectomy and splenectomy in 1 patient and transverse colectomy in 1 patient. The Roux-en Y reconstruction technique was performed after gastrectomy in all the patients who underwent gastrectomy. The median operative time was 295 min and the median blood loss during surgery was 970 ml. The median duration of hospital stay after surgery was 21 days. Postoperative complications developed in 6 patients and the overall morbidity rate was 40%. Pancreatic fistula developed in 2 patients, liver infarction in 1 patient, liver dysfunction in 1 patient, abdominal abscess in 1 patient, bowel obstruction in 1 patient and peritoneal paralysis in 1 patient.

A repeat operation was performed in 1 patient with suspected liver infarction, and cholecystectomy and reconstruction of the hepatic artery were performed for the patient. One patient (6.7%) died of liver failure three months after surgery due to progressive disease of hepatitis C liver cirrhosis. Out of the 11 patients who underwent curative-surgery, 8 received adjuvant chemotherapy (oral S-1 after surgery).

Table II. *Adverse events (n=18).*

	Grade 1 No. (%)	Grade 2 No. (%)	Grade 3 No. (%)	Grade 4 No. (%)
Non hematological toxicity				
Alopecia	3 (16.7)	3 (16.7)	0 (0)	0 (0)
Fatigue	5 (27.8)	3 (16.7)	0 (0)	0 (0)
Anorexia	2 (11.1)	1 (5.6)	3 (16.7)	0 (0)
Nausea	7 (38.9)	2 (11.1)	2 (11.1)	0 (0)
Stomatitis	3 (16.7)	0 (0)	0 (0)	0 (0)
Hematological toxicity				
Leukocytopenia	0 (0)	8 (44.4)	6 (33.3)	1 (5.6)
Neutropenia	1 (5.6)	4 (22.2)	6 (33.3)	5 (27.8)
Anemia	1 (5.6)	5 (27.8)	2 (11.1)	0 (0)
ALT	2 (11.1)	0 (0)	0 (0)	0 (0)

National Cancer Institute Common Toxicity Criteria Version 2.0; ALT: alanine aminotransferase.

Table III. *Tumor response to chemotherapy (n=18).*

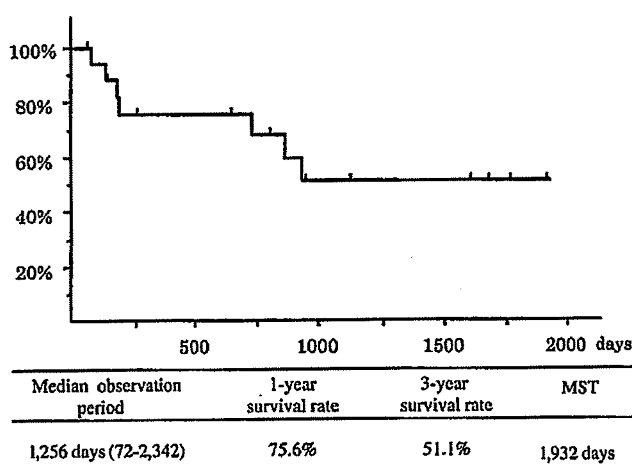
	No (%)				
	CR	PR	SD	PD	RR
Overall	0 (0)	8 (44.4)	8 (44.4)	2 (11.1)	44.4%
Metastases					
LN (17)	0 (0)	8 (47.1)	9 (52.9)	0 (0)	47.1%
Liver (3)	0 (0)	2 (66.7)	0 (0)	1 (33.3)	66.7%
Lung (2)	0 (0)	0 (0)	2 (100)	0 (0)	0%
Peritoneal (1)	0 (0)	0 (0)	1 (100)	0 (0)	0%
Histological type					
Intestinal (6)	0 (0)	2 (33.3)	3 (50.0)	1 (16.7)	33.3%
Diffuse (12)	0 (0)	6 (50.0)	5 (41.7)	1 (8.3)	50.0%

Evaluated by RECIST, CR: complete response, PR: partial responses, SD: stable disease, PD: progressive disease, RR: response rate, LN: lymph node metastasis.

Survival. The median survival had not been reached after a median follow-up of 40 months. The 1- and 3-year survival rates were 75.6% and 51.1%, respectively. Figure 2 depicts the survival curve of all 18 patients calculated by the Kaplan-Meier method.

Discussion

The phase III V325 trial showed that DCF therapy had significant benefits for OS, time to progression and response rate compared to the CF therapy but as mentioned, grade 3 to 4 toxicity occurred in many of the patients (82%). In a Swiss randomized phase II trial, the trio therapy was modified as docetaxel 75 mg/m^2, CDDP 75 mg/m^2 on day 1 plus 5-FU divided into 1-14 days



Median observation period	1-year survival rate	3-year survival rate	MST
1,256 days (72-2,342)	75.6%	51.1%	1,932 days

Figure 2. *Overall survival curve calculated by the Kaplan-Meier method for all 18 patients enrolled in this study.*

infusion of 300 mg/m^2 (33). Grade 3 or 4 neutropenia occurred in 80% and febrile neutropenia in 41% of the patients compared to 29% in the V325 trial. According to these results, we considered that the triumvirate therapy should be modified to a reduced dosage form especially for induction chemotherapy before surgery. Two late phase II trials performed in Japan recommended that docetaxel should be administered intravenously at a dose of 60 mg/m^2 every 3-4 weeks (23, 24). Therefore, the dose of docetaxel was reduced to 60 mg/m^2 and low-dose continuous 5-FU and CDDP was selected. With these modifications, the incidence of grade 3/4 neutropenia and non-hematological toxicity decreased to 61.1% and 27.8%, respectively. Furthermore, febrile neutropenia was only noted in 5.6% of the patients. The adverse events in the present study were acceptable and no treatment-related death was observed. However, one patient with hepatitis C-related liver cirrhosis died three months after surgery. The patient initially recovered after surgery, but the disease status of liver failure progressed after that, suggesting a possible association with the induction chemotherapy. The less toxic regimen showed an overall response rate (PR and CR) by RECIST of 44.4% in the 18 patients. The lymph node and liver metastases showed higher responses 47.1% and 66.7% of the affected patients respectively, but the lung metastases in the two affected patients showed no response. The response rate was in concordance with the reported rate of 36.6% in the Swiss trial (33) and 37% in the V325 study (27).

In conclusion, along with excellent efficacy and moderate toxicity, the reduced dose combination chemotherapy of docetaxel, 5-FU and CDDP is feasible as an induction chemotherapy for patients with AGC.

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Received April 24, 2009
Revised July 6, 2009
Accepted July 17, 2009

INTERNATIONAL JOURNAL OF ONCOLOGY 35: 265-271, 2009

The feasibility of using biopsy samples from esophageal cancer for comprehensive gene expression profiling

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Received January 22, 2009; Accepted March 30, 2009

DOI: 10.3892/ijo_00000336

Abstract. Advanced esophageal cancer has been recently treated by multimodal therapy including preoperative chemotherapy or chemoradiotherapy and surgery. A biopsy sample provides a valuable specimen for understanding the biological characteristics of individual esophageal cancer. Pretreatment prediction of the response to chemotherapy or radiotherapy based on biological characteristics using biopsy samples is a desirable goal. In using biopsy samples for molecular analysis, there are two problems; the proportion of cancer cells and the intratumor heterogeneity. This study was conducted to investigate the feasibility of using endoscopic biopsy samples of esophageal squamous cell cancer (ESCC) for comprehensive gene expression profiling (GEP). Comprehensive GEP was performed in 40 bulky ESCC specimens and 10 normal esophageal epithelial specimens from patients who underwent esophageal resection and 52 endoscopic ESCC biopsy samples from 26 patients (two samples per one patient). Unsupervised hierarchical cluster analysis showed distinct profiles between the bulky ESCC specimens and normal epithelial specimens. Also, unsupervised hierarchical cluster analysis revealed distinct profiles between the biopsy ESCC samples and normal epithelial specimens. Moreover, a couple of biopsy samples taken from different locations of the same tumor were closely clustered together. That is, biopsy ESCC samples were distinguished from normal esophageal epithelial specimens and the intratumor heterogeneity of GEP was smaller than intertumor heterogeneity. GEP using biopsy ESCC samples is feasible and has the potential to represent the biological properties.

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Key words: esophageal cancer, endoscopic biopsy sample, gene expression profiling, intratumor heterogeneity

Introduction

Advanced esophageal cancer, which has a poor prognosis, has been previously treated by multimodal therapy including preoperative chemotherapy or chemoradio therapy and surgery (1,2). Neoadjuvant therapy has been shown to improve the prognosis of responders. On the other hand, non-responders not only suffer from side effects but also lose precious time to take advantage of other possible treatments (3,4). Therefore, pretreatment prediction of the response to chemo- or radiotherapy is one of the most desirable goals in clinical practice, but pretreatment clinocopathological factors are unable to predict the response and there is no reliable method. Biological characteristics of a tumor are important factors affecting the malignant potential and sensitivity to chemotherapy or radiotherapy.

A pretreatment biopsy sample provides a valuable specimen for understanding the biological characteristics of individual esophageal cancer. Molecular analyses, such as RT-PCR and immunohistochemistry, of pretreatment endoscopic biopsy samples of esophageal cancer have been performed to understand the biological characteristics of esophageal cancer (5-7). However, only one gene or a few genes have been addressed in these studies. Multiple genetic alterations are involved in the development and progression of esophageal cancer and these aberrations may affect the expression of a large number of genes (8,9) and numerous molecular pathways may contribute to the sensitivity of chemotherapy or radiotherapy. Gene expression profiling (GEP) allows assessment of expression of thousands of genes simultaneously and is one of the powerful tools for understanding the biological characteristics of each tumor. In fact, this approach has already been used to identify genes that could serve as molecular markers of cancer classification and

outcome prediction (10-14). In esophageal cancer, GEP using surgical resection samples has been performed (15-17). However, these results can be used only in the selection of post operative adjuvant therapy or follow-up schedules. To apply the results of GEP to therapeutic planning of esophageal cancer in clinical practice, pretreatment endoscopic biopsy samples should be analyzed. Recently, GEP using not only surgically resection samples but also biopsy samples has been successfully performed (18-21).

Endoscopic biopsy samples are usually small and morphologically esophageal cancer often displays intratumor macroscopic and microscopic heterogeneity. If the biopsy samples used for molecular analysis contain no or few cancer cells, it would not represent the biological characteristics of a tumor. If the gene expression of samples taken from different locations in the same tumor is drastically different, biological classification based on molecular analysis of biopsy samples may not be suitable. That is, in using biopsy samples for molecular analysis, there are two problems; the proportion of cancer cells and the intratumor heterogeneity of gene expression. However, there have been few studies addressing these problems (7).

This study investigated whether biopsy ESCC samples can be distinguished from normal esophageal epithelial specimens by GEP and assessed the intratumor heterogeneity of GEP by analyzing a couple of biopsy samples taken from different locations of the same tumor.

Materials and methods

Patients and clinical samples. Esophageal squamous cell cancer (ESCC) samples were obtained from 40 patients and normal esophageal epithelial specimens from 10 patients who underwent a surgical resection. The clinicopathological characteristics of the resected ESCC specimens are listed in Table I. Normal esophageal epithelial specimens were collected from the area normally stained by the Lugol dye. In addition, a couple of endoscopic biopsy samples of ESCC were obtained from 26 patients and assayed separately (Fig. 1). The clinicopathological characteristics of the biopsy ESCC specimens are listed in Table II. None of the patients received either chemotherapy or radiotherapy before the surgery or endoscopy. Tissue specimens were disrupted in RNAlater (Ambion, Austin, TX) and stored at 4˚C for 1-2 h, then at -80˚C until use. For each biopsy specimen, an adjacent cancer tissue biopsy was given to a pathologist for assessing the presence of cancer and its histology. Routine hematoxylin and eosin- (H&E) stained slides were used. All aspects of this study protocol were performed according to the ethical guidelines set by the committee of the three Ministries of the Japanese Government and a signed consent form was obtained from each subject.

Cellular composition of the biopsy specimens. The cellular composition of biopsy specimens was determined by an evaluation of the cell squares in the H&E-stained slides using light microscopy. These results were recorded as percentages. A total 110 biopsy samples from 45 ESCC patients, partly including patients enrolled in the microarray analysis, were analyzed.

Table I. The clinicopathological characteristics of the resection ESCC specimens.

Characteristics	No. of patients
Gender	
Male/Female	030/10
Age	
Median	64
Tumor location[a]	
Cervical esophagus	1
Upper thoracic esophagus	7
Middle thoracic esophagus	17
Lower thoracic esophagus	15
Pathological T category[a]	
pT1	6
pT2	3
pT3	26
pT4	5
Pathological N category[a]	
pN0	9
pN1	19
pM1(LYM)	12
Pathological disease stage[a]	
pStage I	2
pStage II	11
pStage III	13
pStage IV	14

[a]According to TNM classification.

Extraction and quality assessment of RNA. Total RNA was purified from clinical samples utilizing TRIzol reagent (Invitrogen, San Diego, CA) as described in the accompanying protocol. The integrity of RNA was assessed by Agilent 2100 Bioanalyzer and RNA 6000 LabChip kits (Yokokawa Analytical Systems, Tokyo, Japan). Only high-quality RNA with intact 18S and 28S ribosomal RNA was used for the subsequent analysis. For control reference, 15 RNA samples from normal esophageal epithelial specimens were mixed.

Preparation of fluorescent-labeled aRNA targets and hybridization. The extracted RNA samples were amplified with T7 RNA polymerase using Amino Allyl MessageAmp™ aRNA kit (Ambion) according to the manufacturer's protocol. The quality of each Amino Allyl-aRNA sample was checked by Agilent 2100 Bioanalyzer. Five μg of control and experimental aRNA samples were labeled with Cy3 and Cy5, respectively, mixed and hybridized on an oligonucleotide microarray covering 30,000 human probes (AceGene Human 30K; DNA Chip Research Inc. and Hitachi Software Engineering Co., Ltd., Yokohama Japan). The experimental protocol is available at http://www.dna-chip.co.jp/thesis/AceGeneProtocol.pdf. Thereafter, the microarrays were scanned using the ScanArray 4000 (GSI Lumonics, Billerica, MA).

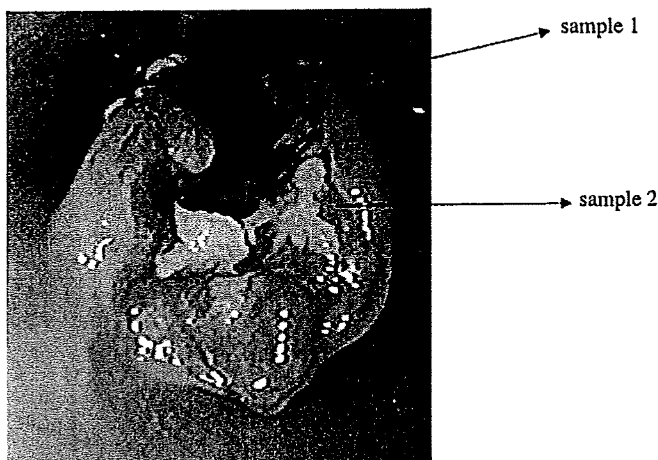

Figure 1. Sampling method for the biopsy ESCC specimens. A couple of biopsy samples were collected from each patient during a routine endoscopic examination.

Table II. The clinicopathological characteristics of the biopsy ESCC specimens.

Characteristics	No. of patients
Gender	
Male/Female	19/7
Age	
Median	67
Tumor location[a]	
Upper thoracic esophagus	11
Middle thoracic esophagus	11
Lower thoracic esophagus	4
Pretherapeutic clinical T category[a]	
cT1	1
cT2	5
cT3	14
cT4	6
Pretherapeutic clinical N category[a]	
cN0	0
cN1	13
cM1(LYM)	13
Pretherapeutic clinical stage[a]	
cStage I	0
cStage II	2
cStage III	9
cStage IV	15

[a]According to TNM classification.

Analysis of microarray data. Signal values were calculated by DNASISArray software (Hitachi Software Inc. Tokyo, Japan). Following background subtraction, data with low signal intensities were excluded from additional investigation. In each sample, the Cy5/Cy3 ratio values were log-transformed and global equalization to remove a deviation of the signal intensity between whole Cy3- and Cy5-fluorescence was performed by subtracting a median of all log(Cy5/Cy3) values from each log(Cy5/Cy3) value. Genes with missing values in >10% of the samples were excluded from further analysis. Hierarchical cluster analysis (HCA) with Euclidean distance as a similarity coefficient and Ward as a clustering algorithm was performed using GeneMath 2.0 software (Applied Maths, Inc., Austin, TX).

Up- or down-regulated genes. Commonly up-regulated genes were defined when their expression levels were 2-fold or more against the control reference in at least 50% of the samples. In addition, commonly down-regulated genes were defined when their expression levels were half-fold or less against the control reference in at least 50% of the samples.

RT-PCR. To verify our microarray data, RT-PCR was performed for two of the commonly up-regulated genes (*MMP9* and *SPARC*). Total RNA (2 μg) from eight biopsy ESCC specimens and control reference (mixture of fifteen RNAs from normal esophageal epithelial specimens) was used for the reverse-transcription reaction with oligo-(dT) primer, using the Reverse Transcription System (Promega, Madison, WI). PCR was performed in a 25 μl reaction mixture containing 1 μl of cDNA template, 0.2 mmol/l of each primer and 1 unit of Taq DNA Polymerase (AmpliTaq Gold, Roche Molecular Systems, Pleasanton, CA), as follows; one cycle of 95°C for 12 min, followed by 25 cycles of 94°C for 1 min, 60°C for 1 min, 72°C for 1.5 min, followed by 72°C for 10 min. The primers were designed by using Web-based Primer 3 software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). GAPDH was also amplified as a marker to confirm the amounts of cDNA generated from each sample. PCR products were visualized with ethidium bromide following separation by electrophoresis on 2% agarose gel.

Results

Cellular composition of biopsy specimens. The mean percentage of tumor cells and stromal cells were 46 and 26% with a standard deviation of 20 and 16, respectively.

Total RNA yield from biopsy specimens. The average and minimum volume of total RNA from one biopsy sample was 17.7 and 2.2 μg, respectively. The quality of all the extracted RNAs was sufficient for comprehensive GEP with intact 18S and 28S ribosomal RNA.

Gene expression profiling between resection ESCC specimens and normal esophageal epithelial specimens and between biopsy ESCC specimens and normal esophageal epithelial specimens. First, GEP was compared between resection ESCC specimens and normal esophageal epithelial specimens. After gene processing described previously, 18,718 genes were used for further analysis. Unsupervised HCA using all 18,718 genes showed distinct profiles between the two groups (Fig. 2). All resection ESCC specimens were distinguished from normal esophageal epithelial specimens by GEP.

Secondly, GEP was compared between biopsy ESCC specimens and normal esophageal epithelial specimens. After gene processing, 18,734 genes were used for further analysis.

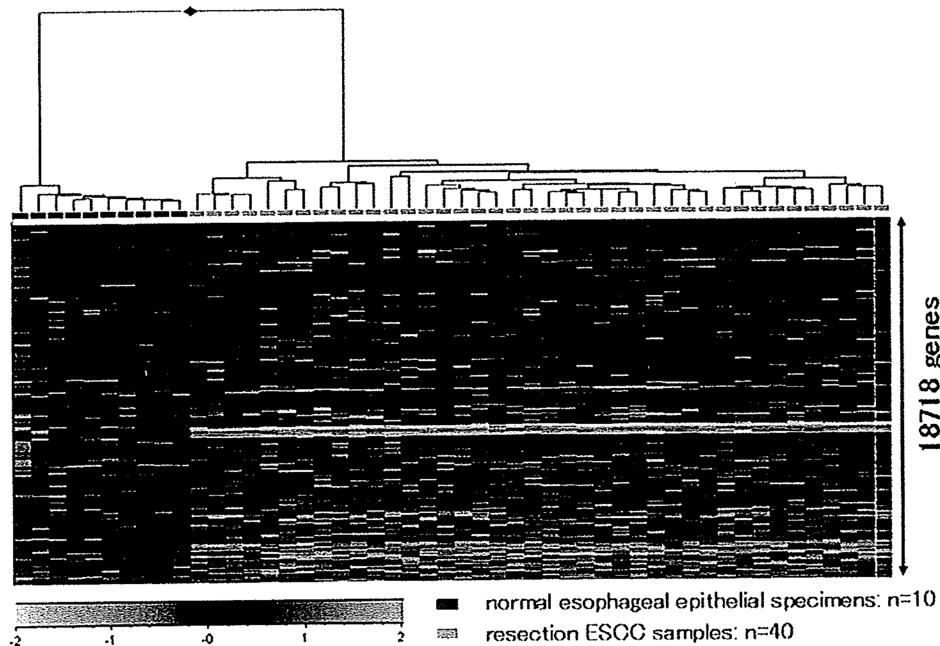

Figure 2. Hierarchical cluster analysis with 18,718 genes in 40 resection ESCC samples and 10 normal esophageal epithelial specimens. The rows and columns represent genes and samples, respectively. The color scale at the bottom indicates the relative expression levels in terms of standard deviations from the median.

Unsupervised HCA using all 18,734 genes showed distinct profiles between the two groups (Fig. 3). Almost all biopsy ESCC specimens except one specimen were distinguished from normal esophageal epithelial specimens by GEP. Moreover, a couple of biopsy samples taken from different locations of the same tumor were closely clustered together, except one case.

Commonly up- or down-regulated genes in resection and biopsy ESCC specimens. A total of 129 and 136 commonly up-regulated genes were identified in the resection and biopsy ESCC specimens, respectively and 85 genes (~65%) were overlapped in both groups. In addition, 518 and 506 commonly down-regulated genes were identified in resection and biopsy ESCC specimens, respectively and 444 genes (~85%) were overlapped in both groups. To confirm the microarray data, RT-PCR was performed for two of the commonly up-regulated genes (*MMP9* and *SPARC*) in eight biopsy samples. These genes have been reported to be associated with progression of esophageal cancer (22,23). The expression patterns of RT-PCR closely agreed with those of the microarray in both genes (Fig. 4).

Discussion

To understand the biological characteristics of individual esophageal cancer, molecular analysis of pretreatment endoscopic biopsy samples have been performed. Miyata *et al* performed immunohistochemical analysis of six molecules in pre radiation biopsy samples. The sensitivity of radiation therapy was significantly correlated with *p53* and *CDC25B* expression (6). Langer *et al* investigated expression of 12 molecules in pretreatment biopsy samples using a real-time RT-PCR analysis and compared the histological effect to cisplatin and 5-fluorouracil chemotherapy. *MTHFR*, *caldesmon* and *MRP1* were significantly associated with the response (7). However, it is clear that several genes will not define the biological characteristics of individual tumors. The properties of each tumor are likely to reflect the functions of all gene products. Therefore, multiple markers will be needed to adequately define the sensitivity of tumors to chemotherapy or radiotherapy and GEP, which can assess the expression of thousands of genes, will likely to be a suitable approach. This is a feasibility study of using biopsy samples in comprehensive GEP for future clinical application.

Cancer tissues consist of mixed populations of cancer cells and stromal cells, such as fibroblasts, infiltrating lymphocytes and endothelial cells. GEP of cancer is currently based on two main methods of RNA preparation; whole tissue RNA extraction and laser capture microdissection (LCM). LCM certainly can improve tissue sampling and achieve homogeneity of the tumor tissue. However, stromal elements play multiple roles in tumor growth and progression and also contribute to tumor response to chemotherapy or radiotherapy (24-26). The biological characteristics of a tumor are considered to be reflected by both the cancer cells and stromal cells, so whole tissues of the specimens were analyzed.

Endoscopic ESCC biopsy samples are small, so it is very difficult to assess the proportion of cancer cells by investigating a part of a sample. In the H&E-stained slides, the average ratio of cancer cells and stromal cells of biopsy specimens was 46 and 26%, respectively, although these samples were different from those actually used in the microarray analysis. If the biopsy sample using comprehensive GEP is composed of mostly normal cells, it would not represent the biological characteristics of a tumor. First, this study confirmed that resection ESCC specimens were distinguished from normal esophageal epithelial specimens by GEP. Then, it verified that biopsy ESCC specimens were distinguished from normal esophageal epithelial specimens by GEP. In this study, the

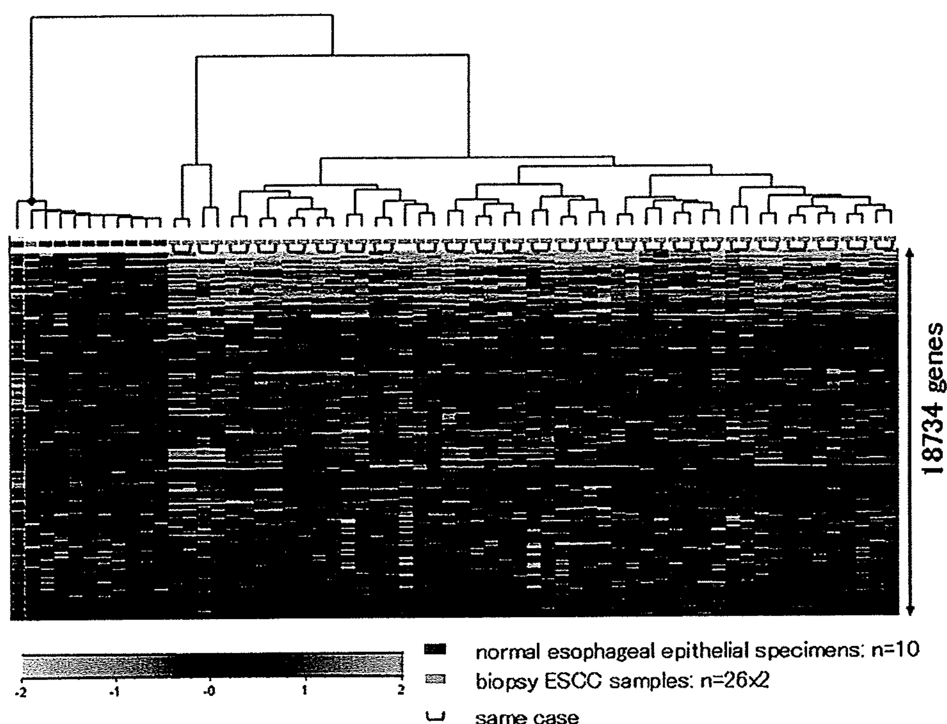

Figure 3. Hierarchical cluster analysis with 18,734 genes in 52 biopsy ESCC samples and 10 normal esophageal epithelial specimens. The rows and columns represent genes and samples, respectively. The color scale at the bottom indicates the relative expression levels in terms of standard deviations from the median.

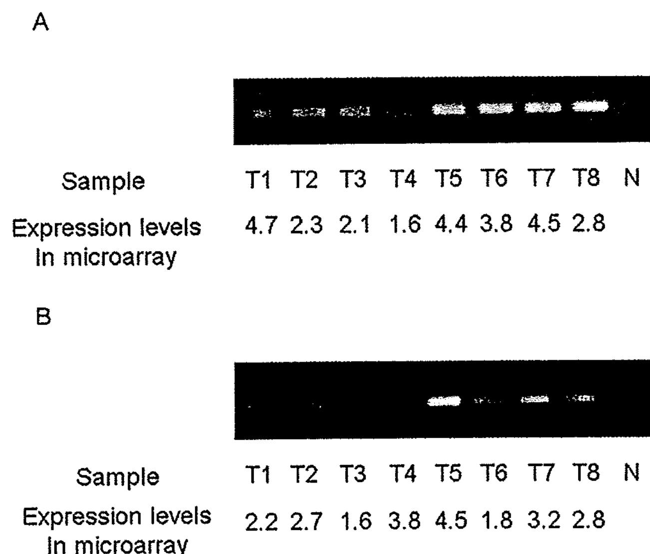

Figure 4. The expression patterns of RT-PCR. (A) *SPARC* and (B) *MMP9*. T1-T8, biopsy cancer sample. N, control reference.

reason that one biopsy specimen could not be distinguished from the normal esophageal epithelial specimens, perhaps, was that there was a low proportion of cancer cells due to a sampling error, though it was possibly adequate for the diagnosis of cancer by microscopy.

There is histopathological heterogeneity in esophageal cancer, as in other solid tumors. The influence of such morphological intratumor heterogeneity on GEP is not clear, though the heterogeneity is detected in individual genes (4,27,28). Therefore, the difference of GEP between biopsy samples taken from different locations in the same tumor should be elucidated. In the current study, a couple of endoscopic biopsy samples obtained from the same case were closely clustered together in almost all cases. This result means that the intratumor heterogeneity of GEP is smaller than intertumor heterogeneity in the superficial position of esophageal cancer and one biopsy specimen may represent GEP of the superficial position of esophageal cancer. Concerning the intratumor heterogeneity of GEP in other solid tumors, the degree of GEP variability within gastric cancer samples isolated from resection specimens of same patient was remarkably low (29). In surgically resected soft tissue sarcomas, the average intratumor distance was considerably shorter than the intertumor distance and intratumor heterogeneity seems to have only a small impact on the variability of GEP (30,31). In endoscopic biopsy samples of colorectal cancer, the intratumor heterogeneity of GEP is smaller than intertumor heterogeneity (21). The findings of these studies are consistent with the current results. It is not clear how many biopsy samples from one patient are sufficient to assess the intratumor heterogeneity. In normal rectal epithelial specimens, two biopsy samples per person are recommended for microarray analysis based on the variation in gene expression data within a person (32). In cervical cancer, although the majority of genes are expressed relatively uniformly, a subset of genes can be expressed quite variably within a single patient. Genes which have a wide variation within a single patient require several biopsies, sometimes >10 biopsies, based on a statistical analysis. However, the optimum number of biopsies cannot be chosen based on statistical reasoning alone, because in the clinical practice, the feasibility of taking many biopsies from one patient is a restrictive factor (33).

The question remains as to whether an endoscopic biopsy ESCC sample reflects the characteristics of the whole tumor. In breast cancer, by comparing GEP of tissue samples with the same cases of FNAB samples, the differences are looked closer (34). Komori *et al* reported that an endoscopic biopsy sample of colorectal cancer might give an accurate picture of the GEP in the whole tumor (21). In this study, although biopsy samples and resection samples were taken from different patients, ~65% of the commonly up-regulated genes and 85% of the commonly down-regulated genes were overlapped. This result indicates that the GEP of endoscopic biopsy samples of ESCC may potentially represent the GEP of whole tumors.

In summary, comprehensive GEP using biopsy ESCC specimens is feasible and has the potential to represent the biological properties of ESCC. Further studies with comprehensive GEP using biopsy samples would provide a novel prediction system of neoadjuvant chemotherapy or chemoradiotherapy for ESCC.

Acknowledgements

We would like to thank Ms. Masayo Yamauchi for her valuable technical assistance. This study was partly supported by the Yasuda Medical Research Foundation.

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Am J Physiol Endocrinol Metab 297: E802–E811, 2009.
First published July 14, 2009; doi:10.1152/ajpendo.00205.2009.

A mouse model of ghrelinoma exhibited activated growth hormone-insulin-like growth factor I axis and glucose intolerance

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Submitted 27 March 2009; accepted in final form 13 July 2009

Iwakura H, Ariyasu H, Li Y, Kanamoto N, Bando M, Yamada G, Hosoda H, Hosoda K, Shimatsu A, Nakao K, Kangawa K, Akamizu T. A mouse model of ghrelinoma exhibited activated growth hormone-insulin-like growth factor I axis and glucose intolerance. *Am J Physiol Endocrinol Metab* 297: E802–E811, 2009. First published July 14, 2009; doi:10.1152/ajpendo.00205.2009.—Ghrelin is a stomach-derived peptide that has growth hormone-stimulating and orexigenic activities. Although there have been several reports of ghrelinoma cases, only a few cases have elevated circulating ghrelin levels, hampering the investigation of pathophysiological features of ghrelinoma and chronic effects of ghrelin excess. Furthermore, standard transgenic technique has resulted in desacyl ghrelin production only because of the limited tissue expression of ghrelin *O*-acyltransferase, which mediates acylation of ghrelin. Accordingly, we attempted to create ghrelin promoter SV40 T-antigen transgenic (GP-Tag Tg) mice, in which ghrelin-producing cells continued to proliferate and finally developed into ghrelinoma. Adult GP-Tag Tg mice showed elevated plasma ghrelin levels with preserved physiological regulation. Adult GP-Tag Tg mice with increased plasma ghrelin levels exhibited elevated IGF-I levels despite poor nutrition. Although basal growth hormone levels were not changed, those after growth hormone-releasing hormone injection tended to be higher. These results indicate that chronic elevation of ghrelin activates GH-IGF-I axis. In addition, GP-Tag Tg mice demonstrated glucose intolerance. Insulin secretion by glucose tolerance tests was significantly attenuated in GP-Tag Tg, whereas insulin sensitivity determined by insulin tolerance tests was preserved, indicating that chronic elevation of ghrelin suppresses insulin secretion and leads to glucose intorelance. Thus, we successfully generated a Tg model of ghrelinoma, which is a good tool to investigate chronic effects of ghrelin excess. Moreover, their characteristic features could be a hint on ghrelinoma.

ghrelin; glucose metabolism

GHRELIN is a stomach-derived 28-amino acid (AA) peptide hormone with octanoyl modification of third Ser residue, which is essential for its binding to growth hormone (GH) secretagogue receptor (GHS-R) (20). There have been several reports regarding ghrelin-producing tumors (9, 17, 36, 37). As far as we know, only two cases have elevated plasma ghrelin level (9, 36). However, the ghrelin-producing cells in the stomach, known as X/A-like cells, account for about 20% of the endocrine cell population in the oxyntic glands (10). It may be reasonable to estimate that far more ghrelinoma cases have been overlooked and diagnosed as nonfunctioning tumors. Hormone-producing tumors demonstrate their characteristic symptoms by chronic effects of each hormone, which may be a key symptom to making a correct diagnosis. Conversely, the characteristic symptom often tells us the chronic effects of each responsible hormone. Acute effects of ghrelin have been studied extensively by many researchers, and a wide variety of acute effects of ghrelin have been discovered, such as the regulation of growth hormone (GH) release, food intake, gastric acid secretion, gastric motility, blood pressure, and cardiac output (23, 25, 26, 31, 33, 34). However, chronic effects of ghrelin have not been fully understood.

To understand the chronic effects of ghrelin, genetically engineered mouse models would be useful. Several groups, including ours, have developed transgenic animals in which ghrelin transgenes are driven by several different promoters (2, 4, 18, 29, 38, 41). All of these animals except for one line created by Reed et al. (29) using the neuron-specific enolase (NSE) promoter and another line recently reported by Bewick et al. (5) using the bacterial artificial chromosome produced only desacyl ghrelin rather than acylated ghrelin. Until the recent identification of ghrelin *O*-acyltransferase (GOAT), which mediates ghrelin octanoylation (40), it had been unclear how acylation of ghrelin takes place. GOAT is expressed mainly in stomach and intestine, and a small amount of GOAT is also present in pancreas (12). This limited expression area of GOAT made it impossible to create ghrelin-overproducing transgenic animals by standard procedures. When we started this study, GOAT had not yet been identified. Accordingly, we choose an approach in which an increase in the number of ghrelin-producing cells in mice would result in increased levels of circulating ghrelin. By taking this approach, we successfully obtained ghrelin promoter-SV40 T-antigen transgenic (GP-Tag Tg) mice. In these mice, ghrelin concentration elevates with age in concordance with the proliferation of ghrelin cells. The aim of this study was to elucidate the pathophysiological features of ghrelinoma and the chronic effects of ghrelin elevation.

MATERIALS AND METHODS

Animals. Two types of fusion genes comprising the 5′-flanking region of human ghrelin gene (4,085 or 1,479 bp) (19) and SV40 T-antigen were designed (Fig. 1*A*). The purified fragments (10 μg/ml) were microinjected into the pronucleus of fertilized C57/B6 mouse (SLC, Shizuoka, Japan) eggs. The viable eggs were transferred into the oviducts of pseudopregnant female ICR mice (SLC) by using standard techniques. Transgenic founder mice were identified by

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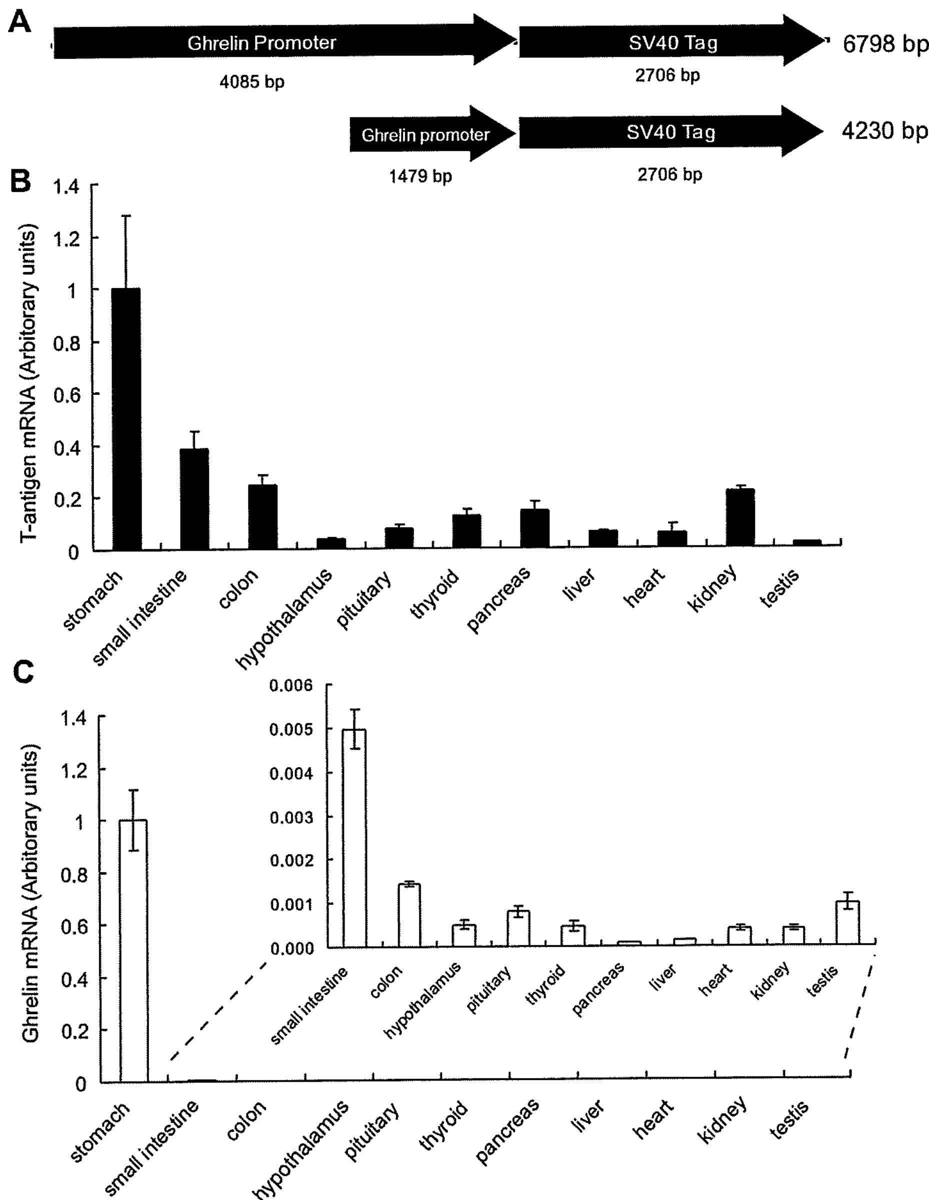


Fig. 1. Constructs of ghrelin promoter-SV40 T-antigen transgenic (GP-Tag Tg) mice and the expression levels of SV40 T-antigen mRNA in various tissues. *A*: 2 types of fusion genes comprising 5′-flanking region of human ghrelin gene (4,085 or 1,479 bp) and SV40 Tag were designed. *B*: the expression levels of SV40 T-antigen mRNA in various tissues of GP-Tag Tg mice at 6 wk of age (n = 8). SV40 T-antigen mRNA was most abundant in the stomachs of GP-Tag Tg mice. *C*: the expression levels of ghrelin mRNA in various tissues of nontransgenic littermates at 6 wk of age (n = 4).

Southern blot analysis of tail DNAs. Transgenic mice were used as heterozygotes. Animals were maintained on standard rodent food (CE-2, 352 kcal/100 g; Japan CLEA, Tokyo, Japan) on a 12:12-h light-dark cycle unless otherwise indicated. All experimental procedures were approved by the Kyoto University Graduate School of Medicine Committee on Animal Research.

RT-PCR and real-time quantitative RT-PCR. Total RNA was extracted using a Sepasol RNA kit (Nacalai Tesque, Kyoto, Japan). Reverse transcription was performed with a high-capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA). RT-PCR was carried out with a GeneAmp 9700 using primers in Table 1 with AmpliTaq Gold PCR master mix (Applied Biosystems). Real-time quantitative PCR was performed using an ABI PRISM 7500 Sequence Detection System (Applied Biosystems) with primers and TaqMan probes or with Power SybrGreen (presented in Table 1). The mRNA expression in each gene was normalized to levels of 18S ribosomal RNA.

Immunohistochemistry. Formalin-fixed, paraffin-embedded tissue sections were immunostained using the avidin-biotin peroxidase complex method (Vectastain "ABC" Elite kit; Vector Laboratories, Burlingame, CA), as described previously (18). Sections were incubated with anti-COOH-terminal ghrelin (AA 13-28) (1:2,000 at final dilution), anti-NH_2-terminal ghrelin (14) that recognizes the n-octanoylated portion of ghrelin (AA 1-11) (1:5,000), anti-glucagon (1:500; DAKO, Glostrup, Denmark), anti-somatostatin (1:500; DAKO), anti-gastrin (1:500; DAKO), and anti-GH (1:500; DAKO). The cell number of ghrelin-immunopositive cells was analyzed by WinRoof visual analysis software (Mitani, Fukui, Japan).

Measurements of plasma and tissue ghrelin concentrations. Collection of plasma samples was performed as reported previously (18). Plasma ghrelin and desacyl ghrelin concentrations were determined using two separate ELISA kits, an active ghrelin ELISA kit that recognizes n-octanoylated ghrelin and a desacyl ghrelin ELISA kit (both from Mitsubishi Kagaku Iatron, Tokyo, Japan) (1). Tissue ghrelin concentration was determined by radioimmunoassay (RIA) using anti-ghrelin (AA 13-28) antiserum (C-RIA) and anti-ghrelin (AA 1-11) antiserum (N-RIA), as described previously (18).

Western blot. Stomachs were boiled for 5 min in the 10-fold vol/wt of water. Acetic acid was added to each solution so that the final concentration was adjusted to 1 M, and the tissues were homogenized.

Table 1. *PCR primers and TaqMan probes*

Gene	Primer Sequence
Ghrelin	
Sense	5′-GCATGCTCTGGATGGACATG-3′
Antisense	5′-TGGTGGCTTCTTGGATTCCT-3′
TaqMan probe	5′-AGCCCAGAGCACCAGAAAGCCCA-3′
NPY	
Sense	5′-TCCGCTCTGCGACACTACAT-3′
Antisense	5′-GGAAGGGTCTTCAAGCCTTGT-3′
TaqMan probe	5′-CAAGGGCTGGATCTCTTGCCATATCTCTG-3′
AgRP	
Sense	5′-GCTCCACTGAAGGGCATCA-3′
Antisense	5′-TAGCACCTCCGCCAAAGCT-3′
TaqMan probe	5′-TTCCCAGGTCTAAGTCTGAATGGCCTCA-3′
GHRH	
Sense	5′-AGGATGCAGCGACACGTAGA-3′
Antisense	5′-TCTCCCCTTGCTTGTTCATGA-3′
TaqMan probe	5′-CCACCAACTACAGGAAACTCCTGAGCCA-3′
Somatostatin	
Sense	5′-AGCTGAGCAGGACGAGATGAG-3′
Antisense	5′-ACAGGATGTGAATGTCTTCCAGTT-3′
TaqMan probe	5′-CGAACCCAGCAATGGCACCCC-3′
GHS-R	
Sense	5′-CACCAACCTCTACCTATCCAGCAT-3′
Antisense	5′-CTGACAAACTGGAAGAGTTTGCA-3′
TaqMan probe	5′-TCCGATCTGCTCATCTTCCTGTGCATG-3′
GH	
Sense	5′-AAGAGTTCGAGCGTGCCTACA-3′
Antisense	5′-GAAGCAATTCCATGTCGGTTC-3′
TaqMan probe	5′-CCATTCAGAATGCCCAGGCTGCTTTC-3′
GHRH-R	
Sense	5′-GCCCTTGGAACTGTTAACCA-3′
Antisense	5′-GCAACCAGGATGGCAATAGC-3′
TaqMan probe	5′-AGCATCTCCATTGTAGCCCTCTGCGTG-3′
SV40 Tag	
Sense	5′-AAACACTGCAGGCCAGATTT-3′
Antisense with power SYBR Green	5′-AAATGAGCCTTGGGACTGTG-3′
PC1/3	
Sense	5′-AGTGGAAAAGATGGTGAATG-3′
Antisense	5′-CTCCTCATTTAGGATGTCCA-3′

NPY, neuropeptide Y; AgRP, agouti-related protein; GHRH, growth hormone (GH)-releasing hormone; GHS-R, GH secretagogue receptor; GHRH-R, GHRH receptor; PC1/3, prohormone convertase 1/3.

The supernatant was loaded onto a Sep-Pak C18 cartridge (Waters, Milford, MA) preequilibrated with 0.9% NaCl after centrifugation. The cartridge was washed with 2.5 ml of 5% CH_3CN-0.1% trifluoroacetic acid and eluted with 2.5 ml 60% CH_3CN-0.1% trifluoroacetic acid. The eluate was evaporated, lyophilized, and dissolved in Novex Tricine SDS Sample Buffer (Invitrogen, Carlsbad, CA). After being heated at 85°C for 2 min, 20 mg of samples of initial weight were subjected to tricine-SDS PAGE and electroblotted to polyvinylidene fluoride membranes (Invitrogen). Transferred membranes were blocked with Immunoblock (Dainippon Seiyaku, Osaka, Japan) and then incubated with anti-COOH-terminal ghrelin antibody (1:5,000). After being washed with PBS-0.1% Tween-20, membranes were reacted with secondary antibodies and developed with ECL plus (GE Healthcare, Buckinghamshire, UK) as instructed by the manufacturer. The signal on the blot was detected with Lumino-Image Analyzer LAS-3000 mini system (Fuji Photo Film, Tokyo, Japan).

Measurement of food intake. Mice were housed individually with continuous access to chow and water. Food intakes were measured by subtracting the remaining weight of the chow from that originally presented. As for measuring the food intake by ghrelin, ad libitum-fed mice were injected with ghrelin (120 or 360 μg/kg) or saline subcutaneously. Food intakes were measured for 2 h after injection.

Measurements of lean body mass, fat mass, and bone mass. Mice were anesthetized with pentobarbital sodium. Lean body mass, fat mass, and bone mass of mice were measured by an animal computed tomography system (Latheta LTC-100; Aloka, Tokyo, Japan).

Measurements of hormones and blood glucose levels. Serum GH levels were determined by a rat GH EIA kit (SPI Bio, Massy Cedex, France). Serum insulin-like growth factor I (IGF-I) levels were measured using a mouse IGF-I immunoassay kit (R & D Systems, Minneapolis, MN). Blood glucose levels were determined by glucose oxidase method using Glutest Sensor Neo (Sanwa Kagaku, Kyoto, Japan). Measurement of serum insulin concentrations was performed by ELISA using an ultrasensitive rat insulin kit (Morinaga, Yokohama, Japan).

GH-provocative test. GH-provocative test was carried out as described previously (16). Serum samples were collected at 15 min after subcutaneous injection of 180 μg/kg of GH-releasing hormone (GHRH) or 120 μg/kg of ghrelin. We choose these doses according to the results of our previous study (16).

Glucose and insulin tolerance tests. For the glucose tolerance test, after overnight fast, the mice were injected with 1.5 g/kg glucose intraperitoneally. For the insulin tolerance test, after a 4-h fast, mice were injected with 1.0 mU/g human regular insulin (Novolin R; Novo Nordisk, Bagsvaerd, Denmark) intraperitoneally. Blood was sampled from the tail vein before and 30, 60, 90, and 120 min after the injection.

Insulin release. After overnight fast, the mice were injected with 3.0 g/kg glucose intraperitoneally. Blood was sampled from the retroorbital vein at 2 and 30 min after the injection using a glass tube.

Statistical analysis. All values were expressed as means ± SE. The statistical significance of the differences in mean values was assessed by repeated-measures ANOVA or Student's *t*-test. The statistical difference in the changes of plasma ghrelin levels by feeding were assessed by paired *t*-test. Pearson's correlation coefficient analysis and simple regression were used to assess the relations between plasma ghrelin level and body weight. Difference of correlation coefficients of the regression lines obtained from GP-Tag Tg mice and nontransgenic littermates was determined by testing the *t* value.

RESULTS

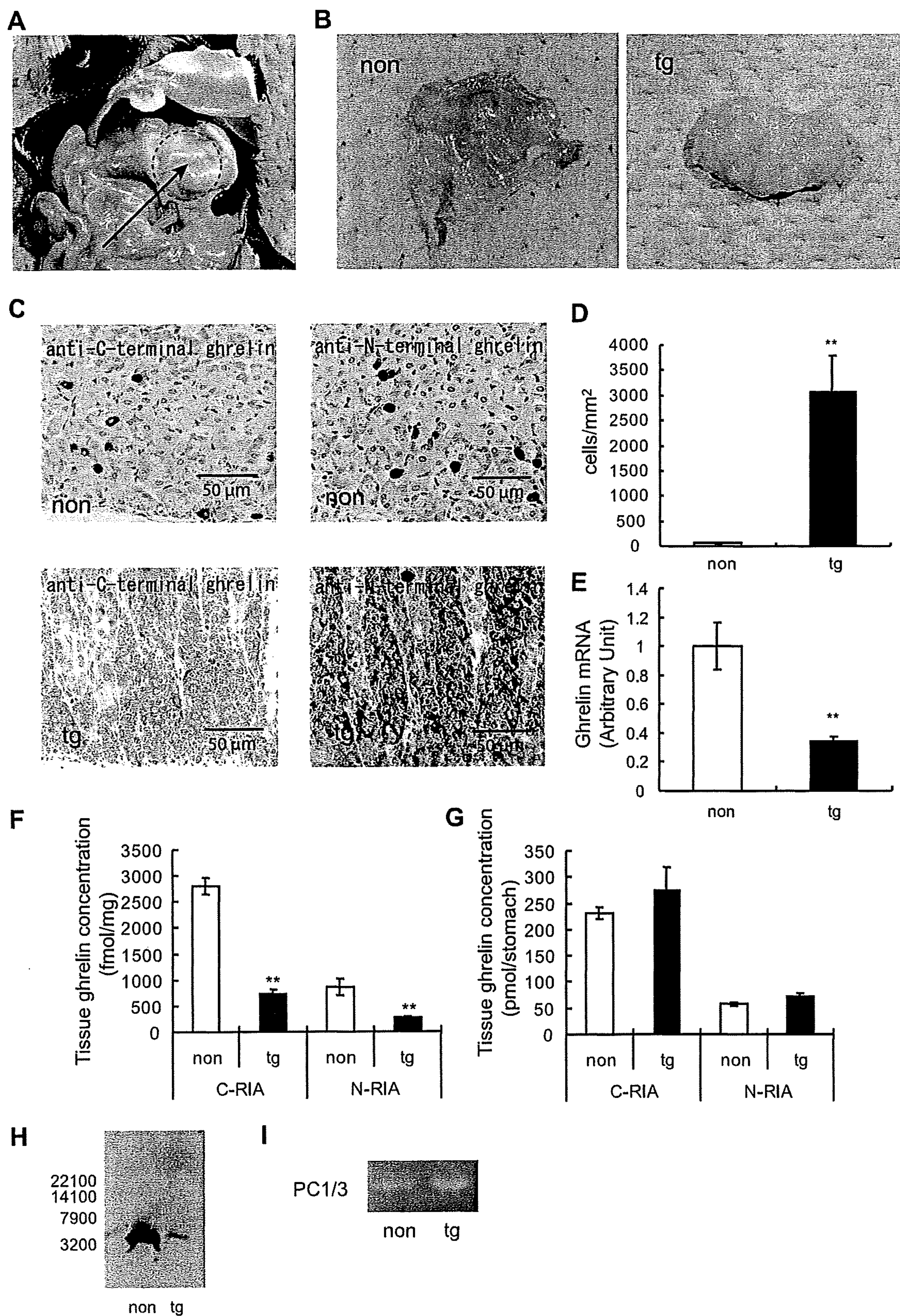
Generation of GP-Tag Tg mice. By injecting transgenes into 846 eggs, we obtained 11 lines of GP (4.85) Tag Tg mouse. We succeeded in breeding three of these lines (1-5, 3-1, and 4-3). Among these three lines, mice of the 3-1 line developed gastric tumor and showed elevated plasma ghrelin levels, as described below. Mice of the 1-5 line showed very aggressive tumor development and died at ~13 wk of age because of thyroid, pancreatic, and gastric tumors. Mice of the 4-3 line showed very slow tumor development. The proliferation of ghrelin cells was

Fig. 2. Pathological findings and tissue ghrelin concentrations of stomachs in GP-Tag Tg mice. *A–C*: macro findings of stomachs in GP-Tag Tg mice (*A*: arrow, dotted area; *B*: Tg) and nontransgenic littermates (non; *B*) at 12 wk of age. Stomach walls of GP-Tag Tg mice were hypertrophic. *C*: immunohistochemical analysis of ghrelin peptide expression in tissue sections of stomachs of GP-Tag Tg mice (Tg) and nontransgenic littermates (non) using anti-COOH-terminal and anti-NH_2-terminal ghrelin antibodies. *D*: the cell number of ghrelin-immunopositive cells in Tg and non littermates. *E*: the mRNA levels of ghrelin in 12-wk-old male Tg mice and non littermates; $n = 5$, $^{**}P < 0.01$ compared with nontransgenic littermates. *F* and *G*: tissue concentration per milligram (*F*) and per stomach (*G*) of ghrelin peptide in 12-wk-old male Tg mice (black bars) and non littermates (open bars); $n = 6$, $^{**}P < 0.01$ compared with non littermates. C-RIA, total ghrelin (ghrelin and desacyl ghrelin); N-RIA, ghrelin. *H*: Western blot analysis of stomach samples of Tg and non littermates using anti COOH-terminal ghrelin antibody. *I*: RT-PCR analysis of prohormone convertase 1/3 (PC1/3) mRNA expression in the stomach of Tg.

modest even at 50 wk of age in the 4-3 line. Accordingly, we analyzed mainly GP-Tag Tg mice of the 3-1 line.

We could not get a transgene-positive mouse of GP (1479) Tag Tg mouse by injecting transgenes into 631 eggs.

The expression levels of SV40-Tag mRNA among various tissues. We first examined the expression levels of SV40-Tag mRNA in various tissues of GP-Tag Tg mice, including stomach, small intestine, colon, hypothalamus, pituitary, thyroid,



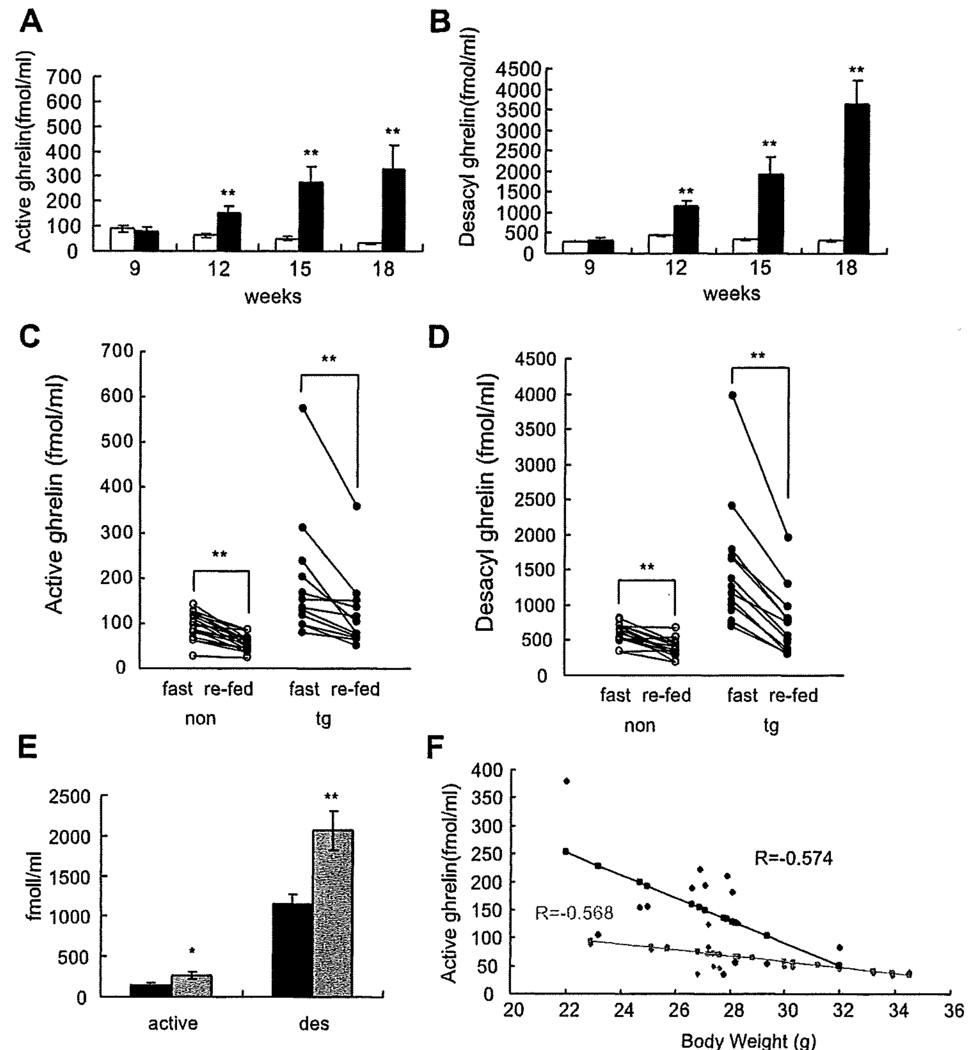


Fig. 3. Plasma ghrelin and desacyl ghrelin levels in GP-Tag Tg mice. *A* and *B*: plasma ghrelin (*A*) and desacyl ghrelin (*B*) levels in male GP-Tag Tg mice (black bars) and nontransgenic littermates (open bars); $n = 3–17$. $^{**}P < 0.01$ compared with non littermates. *C* and *D*: plasma ghrelin (*C*) and desacyl ghrelin (*D*) levels after overnight fasting (fast) and after refeeding (refed) in 15-wk-old male Tg and non mice. $^{**}P < 0.01$; $n = 12–18$. *E*: plasma ghrelin (active) and desacyl ghrelin (des) levels in 12-wk-old male (black bars) and female (gray bars) GP-Tag Tg mice; $n = 7–13$, $^{*}P < 0.05$, $^{**}P < 0.01$ compared with male GP-Tag Tg mice. *F*: plasma ghrelin levels were correlated with body weights in 12-wk-old male GP-Tag Tg mice (black bars; $r = -0.574$, $P < 0.01$) and in nontransgenic littermates (gray bars; $r = -0.568$, $P < 0.05$). The regression coefficient of the regression line of GP-Tag Tg mice was bigger than that of nontransgenic littermates ($t = 2.08$, $P < 0.05$).

pancreas, liver, heart, kidney, and testis (Fig. 1*B*). The highest expression levels were observed in stomach, and the second-highest levels were observed in small intestine. The expression pattern of SV40-Tag mRNA was almost similar to that of ghrelin (Fig. 1*C*).

Pathological feature and tissue ghrelin concentration of stomach of GP-Tag Tg mice. Stomach walls of GP-Tag Tg mice became hypertrophic with age (Fig. 2, *A* and *B*). Immunohistochemical analysis by both anti-COOH-terminal and anti-NH_2-terminal ghrelin antibodies revealed hyperplasia of ghrelin-immunopositive cells (Fig. 2, *C* and *D*), although the staining in GP-Tag Tg mice was paler than that in nontransgenic littermates (Fig. 2*C*). These hyperproliferating cells were not immunostained with anti-glucagon, somatostatin, or gastrin antibodies (data not shown).

The mRNA levels of ghrelin in the stomachs of 12-wk-old male GP-Tag Tg mice were significantly lower than those of nontransgenic littermates ($P < 0.01$, $n = 6$; Fig. 2*E*). Consistent with this observation, tissue concentrations of ghrelin (N-RIA; fmol/mg tissue) and total ghrelin (desacyl ghrelin plus ghrelin) (C-RIA) of 12-wk-old male GP-Tag Tg mice were significantly lower than those of nontransgenic littermates ($P < 0.01$, $n = 6$; Fig. 2*F*). However, since the weights of the stomach of GP-Tag Tg mice were significantly higher than controls (non-Tg vs. Tg, 83.4 vs. 362.0 mg, $P < 0.01$) due to the hypertrophy of the stomach wall, the tissue ghrelin concentration per whole stomach tended to be higher in GP-Tag Tg mice [not significant (NS), $n = 6$; Fig. 2*G*]. The size of ghrelin content of GP-Tag Tg mice was similar to that of nontransgenic littermates when analyzed by tricine-SDS PAGE and Western blot analysis (Fig. 2*H*), indicating that processing of preproghrelin to ghrelin occurred in hyperproliferaing ghrelin cells in GP-Tag Tg mice. The mRNA of prohormone convertase 1/3, which processes preproghrelin to ghrelin, was detected in the stomachs of GP-Tag Tg mice (Fig. 2*I*).

Plasma ghrelin levels of GP-Tag Tg mice. Plasma ghrelin and desacyl ghrelin levels of GP-Tag Tg mice were almost equal to those of nontransgenic littermates at 9 wk of age and then increased with age ($n = 3–17$; Fig. 3, *A* and *B*), with some variations in the levels among animals.

We next examined whether physiological regulation of ghrelin secretion is preserved in GP-Tag Tg mice. Plasma ghrelin and desacyl ghrelin levels of GP-Tag Tg mice were increased by fasting and decreased by refeeding $P < 0.01$, ($n = 7–13$; Fig. 3, *C* and *D*). Plasma ghrelin and desacyl ghrelin levels of female GP-Tag Tg mice were significantly higher than those of